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A Survey of Selected Methods of Arsenic Analysis in Biological Materials and a Proposed Modification of the Evans-Bandemer Method

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A SURVEY OF SELECTED METHODS OF ARSENIC ANALYSIS IN BIOLOGICAL MATERIALS AND A PROPOSED MODIFICATION OF THE EVANS-BANDEMER METHOD

by

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This thesis is approved as a creditable independent investigation by a candidate for the degree, Master of Science, and acceptable as meeting the thesis requirements for this degree, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

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INTRODUCTION

Arsenic (As) atomic number 33, atomic weight 74.91, is usually classed as a nonmetal or metalloid, although in the pure state it forms a steel grey metallic crystal and is referred to as metallic arsenic. Only one stable isotope is known at the present time, in spite of its fractional atomic weight. However, the number of allotropic forms and their properties seem to be undecided among many authors (1).

"Arsenic" was known in the pre-Christian era, but as such was not the pure element. Instead it consisted of the sulfides of arsenic: realgar (As_4S_4) and orpiment (As_2S_3). These sulfides were encountered in the gold, silver, and tin mines of that time, accounting for the deaths of many of the miners. The early investigations of arsenic trioxide (As_2O_3) mentioned its medicinal values, but apparently the toxic effects were not discovered until around the first century A. D. (1, 2).

It is believed that elemental arsenic was obtained by Albertus Magnus in 1250 A. D., by heating As_2S_3 with soap (2).

Perhaps the most important use of arsenic at that time was by the professional poisoners as a means of assassination (3). It is said that many a Roman wife dispatched her unworthy husband by this means.

Today, the United States is the largest commercial producer of arsenic with respect to the ore processing method, and Mexico ranks second. Production was far in excess of demand, and for a time the arsenic produced from ore refining was sealed in cement blocks and dumped at sea. Due to the expense of disposal, however,

storehouses were built with the hope of developing a future market for it. World production in 1943 was about 77,000 tons of white arsenic. In 1939 it sold for about three cents a pound, and by 1947 had doubled in price. In 1944 the United States produced about 36,000 tons and consumed about 43,000 tons (4).

During the past twenty years there has been an increase in arsenic consumption. Insecticides and glass manufacturers utilize about 80 % and 5 % respectively of the white arsenic produced today. In addition, a small percentage is used in metallurgy and drug preparations (4).

The hazards of arsenic are many to the people producing or working with arsenical compounds. They, however, are aware of these dangers. Instead it is the unwary who are hurt by the substance, often through an indirect manner. The official food tolerance as established by the Federal Food and Drug Administration is 3.57 parts per million of As as As_2O_3, or 2.71 parts per million of As as As (5). This is the same as 2.71 micrograms or 0.00000271 gram of arsenic per gram of food. Consequently, there exists a need for a suitable method by which arsenic may be determined.

Food products are often highly contaminated as the result of sprays or insecticides used on foods. Another source of arsenic poisoning is in the areas near ore processing plants. Mineral waters contain small quantities of arsenic due to leaching and weathering of mineral rocks.

Perhaps the most important occurrence of arsenic to the average individual is in the form of foodstuffs and biological materials. A case of arsenic poisoning from beer was traced to the sulfuric

acid used in processing the sugar (6). Wines as well contain traces of arsenic as the result of sprays used on the grapes (7). The problem is important from many standpoints, whether it be glass-making, ore processing, pharmaceutical preparations, foodstuffs, or in the involvement of any of the hundreds of other occurrences of arsenic. It still must be sought out, separated, and determined quantitatively in a satisfactory fashion. The variety of its occurrences makes a universal method of analysis elusive.

The objective of this investigation was to determine the best available chemical method for the determination of arsenic in micro quantities in biological materials, utilizing simple and readily available equipment. The author has attempted to make a survey of the most promising methods of arsenic analysis in biological materials and to improve or devise a better method in accordance with the objective of this problem.

SURVEY OF THE METHODS

There are probably as many methods and variations of methods as there are workers investigating arsenic analysis. This condition would indicate that there is no entirely satisfactory method. Two of the most commonly encountered methods in the textbooks are the Marsh and the Gutzeit tests, although in recent years they have not proved too satisfactory as quantitative tests. The Marsh test depends upon the reduction of the oxide of arsenic to arsine, with its subsequent decomposition and deposition of the elemental arsenic as a mirror (8). The Gutzeit method, the official method of the Association of Official Agricultural Chemists, is based on the generation of arsine which reduces mercuric bromide on a strip of filter paper to produce a brownish-yellow stain, the length of the stain (in mm.) being proportional to the concentration of the arsine generated. Some authors and investigators also use the shade of the stain as a means of analysis (9). The original method called for silver nitrate impregnated strips of filter paper which produced a stain of $AsAg_3 \cdot 3AgNO_3$ (8).

Of the several modifications of the Gutzeit method that have since appeared, the most promising perhaps is that of Almond (10). This investigator placed a coupling in the glass tube which normally contains the indicator paper in such a way that a circular disk of impregnated paper lay horizontally across the path of the escaping arsine. In this method the arsine was forced to pass through the paper and could not escape along the sides of it. Almond used various shades of the stain as standards and claimed an accuracy of one microgram by this modification.

In consulting the literature several methods were encountered, but the most promising seemed to be the Sultzaberger (11). The apparatus required is simple and may be obtained in almost any laboratory, with perhaps the exception of the 25 ml. Fresenius flask. In addition, the method seems to be a rapid one not requiring the usual long waiting period for the generation of arsine. Here the biological material is destroyed by wet combustion using nitric and sulfuric acids, leaving the arsenic in the pentavalent form, probably the oxide. It is then reduced and distilled as the trichloride, being absorbed by dilute nitric acid solution. Evaporation, followed by treatment with ammonium molybdate, produced the molybdiarsenic acid which was reduced to the stable blue colored complex using hydrazine sulfate.

The Kingsley and Schaffert (12) method requires a little more elaborate apparatus, but it was examined in detail because of its rapid hydrochloric acid digestion. The biological material is digested in dilute hydrochloric acid solution for fifteen minutes, treated with 10 ml. of HCl, diluted to the previous volume, treated with 15 % KI solution, and then with 40 % $SnCl_2$. The arsenic is then separated as arsine using mossy zinc and is absorbed by a standard iodine solution. Again the heteropoly blue colored complex is formed by using ammonium molybdate and hydrazine sulfate. The standard curve is prepared by treating known standards of arsenic with the standard iodine, developing the color and reading the percent transmission at 865 mu on the Beckman DU spectrophotometer.

In March, 1954, a modification of the Kingsley method appeared in Analytical Chemistry by Evans and Bandemer (13), in which the

biological material was ashed with a saturated magnesium nitrate solution. The ash was then dissolved in dilute HCl, treated with 15 % KI, then 40 % $SnCl_2$, and arsine was generated by the addition of mossy zinc. The arsine was absorbed by a standard iodine solution, and the blue colored heteropoly complex was developed as before. The wave length was altered to 840 mu on the Beckman DU spectrophotometer, since maximum absorption is obtained from the blue complex at this wave length rather than at 865 mu. Although the reading is most sensitive at the maximum absorption, the main point to be remembered is that the same wave length must be used consistently.

The author then attempted a modification of this method using dilute sulfuric instead of hydrochloric. The blue complex was read at 840 mu on the Beckman DU spectrophotometer, and the red filter was used on the Cenco photelometer.

In addition, there are other methods applicable, such as distillation, titration, and polarography. Most of the methods are time consuming, require elaborate equipment that is not always available, and are not satisfactory for micro quantities of arsenic. A polarographic method was considered and discarded in favor of simplicity.

EXPERIMENTAL PROCEDURES AND RESULTS

In order to become familiar with the techniques of the various methods of determination, it was decided to select a few of the most promising methods of arsenic analysis and apply them to the problem at hand by preparing standard curves and doing recovery experiments. The final test would be an analysis of biological material in which the arsenic was organically bound.

1. The Gutzeit Method (9).

Reagents

Reagents were prepared according to the specifications of the A. O. A. C. with the exception of the zinc, the mercuric bromide paper, and the standard stock arsenic solution.

Zinc - Reagent grade 30 mesh zinc by Mallinckrodt was used directly instead of zinc treated with HCl.

Mercuric bromide paper - Twelve cm. strips of Whatman No. 2 filter paper cut 3 mm. in width were treated with 5 % alcoholic mercuric bromide solution and dried. The papers were prepared as needed.

Standard arsenic solution (11). - The solution was prepared by dissolving 1.32 g. of Bureau of Standards arsenic trioxide in 30 ml. of 1 N sodium hydroxide, diluted, and the pH adjusted to 6.5 - 6.8 using 1 N hydrochloric acid. The pH of the solution was further adjusted to 7.2 using 2 N sodium bicarbonate and diluted to 1 liter. It was then standardized against a standard iodine solution. Ten milliliters of this stock solution diluted to one liter provided a solution that contained 10 micrograms of As as As per ml.

Apparatus

The apparatus used conformed to the directions as given in the

A. O. A. C. The Kjeldahl flask was made from a 500 ml. distillation flask by removing the side arm flush with the neck and sealing the opening.

Procedure

From 0.2 to 0.3 g. of arsenic-free laboratory chow were placed in a 500 ml. Kjeldahl digestion flask, and an appropriate volume of standard arsenic (10 ug./ml.) solution was added. The organic material was then wet ashed with 20 ml. of H_2SO_4 and 35 ml. of HNO_3. Nitric acid was added as required until digestion was complete. The digest should be colorless to a straw yellow at this point.

Seventy five milliliters of distilled water and 25 ml. of saturated ammonium oxalate were added to the cooled digest and the solution then evaporated until SO_3 fumes appeared. The digest was cooled and diluted to 100 ml. in a volumetric flask.

A 20 ml. aliquot (1 - 10 ug. As) was then neutralized with 25 % NaOH. Five milliliters each of concentrated HCl and 15 % KI solution were added together with 4 drops of 40 % $SnCl_2$. The container was placed in a water bath (25^0C.) and allowed to stand 30 minutes. The zinc was then added and the absorption tube containing the indicator paper attached. The reaction was allowed to proceed 1.5 hours.

The indicator papers were removed and the average length of the stain measured in mm. This procedure was followed for standard stains of 1, 3, 5, 7, and 10 ug. of arsenic; in all cases the volume was kept constant. The results may be found in Figure 1 and Table I.

Recovery trials

Recovery trials were attempted with various biological materials, using known quantities of arsenic.

Flour

Five gram samples of milled white flour containing known quantities of arsenic (1 - 10 ug. of As per 20 ml. aliquot) were analysed in duplicate. The samples were digested, diluted to 500 ml., and 20 ml. aliquots neutralized and analyzed according to the procedure given. The results are shown in Table II.

Rat kidney homogenate

A rat kidney homogenate was prepared and a 20 ml. aliquot taken in duplicate. To the aliquot was added 0.5 ml. of standard arsenic solution (5 ug. As), and the samples were treated as described. The results are found in Table III.

Rat liver homogenate

A 20 % rat liver homogenate was prepared and a 20 ml. aliquot taken to which was added 5 ug. of arsenic. Recoveries are shown in Table III.

Erratic results were obtained in the attempt to prepare a standard curve, and after some consideration it was decided to determine the optimum quantity of zinc required. This was done by following the procedure from digestion through the arsine generation using 10 ug. of arsenic. The zinc was then altered from 1.0 to 10.0 g. in 1.0 g. quantities. The results are shown in Table IV.

TABLE I

STANDARDIZATION DATA FOR GUTZEIT METHOD

Sample	1	2	3
Blank	1 mm.	1 mm.	0 mm.
1 ug. As	0 mm.	2 mm.	1 mm.
3 ug. As	4 mm.	3 mm.	4 mm.
5 ug. As	6 mm.	6 mm.	6 mm.
7 ug. As	8 mm.	5 mm.	7 mm.
10 ug. As	10 mm.	5 mm.	10 mm.

TABLE II

ARSENIC RECOVERIES FROM FLOUR BY GUTZEIT METHOD

Sample	1	2	3
Blank	1 mm.	0 mm.	1 mm.
1 ug. As	3 mm.	2 mm.	3 mm.
3 ug. As	6 mm.	4 mm.	3 mm.
5 ug. As	7 mm.	0 mm.	7 mm.
8 ug. As	11 mm.	11 mm.	14 mm.
10 ug. As	12 mm.	11 mm.	12 mm.

TABLE III

ARSENIC RECOVERY FROM ANIMAL TISSUE BY GUTZEIT METHOD

Sample	Trial	Length of Stain (mm.)	ug. of As Recovery
Rat kidney	blank	1	0
homogenate +	1	2	1
5 ug. As added	2	2	1
20 % rat liver	3	5	4
homogenate +	4	5	4
5 ug. As added			

TABLE IV

OPTIMUM ZINC DETERMINATION FOR THE GUTZEIT METHOD

Weight of zinc in grams	Blank Stain (mm.)	Sample I Stain (mm.)	Sample II Stain (mm.)
1.0	0	5.0	4.7
2.0	0	7.1	6.6
3.0	0	6.9	7.0
4.0	0	8.6	8.6
5.0	0	8.9	9.4
6.0	0	9.5	10.6
7.0	0	11.3	11.2
8.0	0	12.2	11.4
9.0	0	13.7	13.8
10.0	trace	15.7	15.7

Results

The data in Figure 1 and Table I show the standardization curve for the Gutzeit method from 1 to 10 ug. of arsenic. At first glance, the curve presents an ideal picture. It was obtained, however, only after repeated trials. Table II, which is essentially the same experiment repeated with flour, presents several inconsistencies.

For example at 3 ug., the stain varies in length from 3 to 6 mm. with the average being about 4 mm. If the blank of 1 mm. is subtracted, this means that 3 ug. of arsenic should produce a stain 3 mm. long.

Recovery on the rat kidney homogenate was poor regardless of the agreement between samples. It demonstrates what might happen to the average analyst making duplicate determinations for micro quantities of arsenic by this method.

Table IV shows the results of an attempt to determine optimum zinc requirements. The optimum quantity of zinc appears to be between 4 and 5 grams. However, the author used 9.0 grams of zinc in the experimental work to insure complete evolution of arsine and still obtain a minimum blank which could be used as a reference.

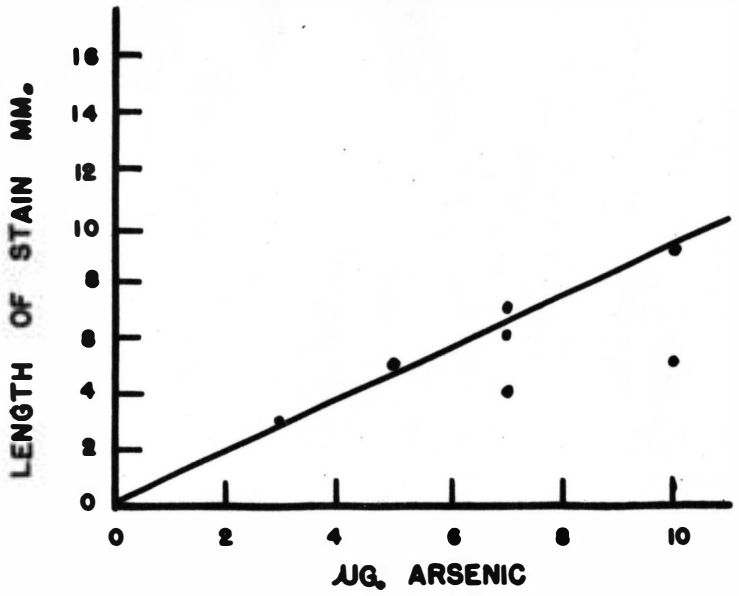

FIGURE 1

PLOT OF STANDARDIZATION CURVE FOR THE GUTZEIT METHOD

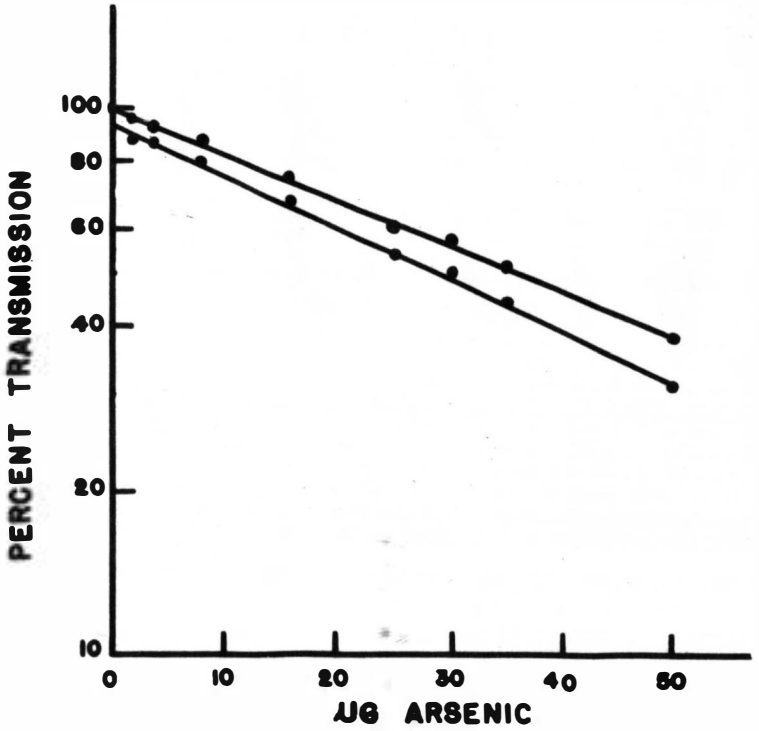

FIGURE 2

PLOT OF STANDARDIZATION CURVE FOR THE SULTZABERGER METHOD

2. The Sultzaberger Method (11)

Reagents and Apparatus

The reagents and apparatus conformed to the specifications as outlined by Sultzaberger.

Procedure

The arsenic-containing biological material was wet ashed using a mixture of sulfuric and nitric acids in a 250 ml. Erlenmeyer flask on a hot plate. Digestion was carried out slowly to prevent excessive foaming and loss of arsenic. As digestion proceeded, the oxidizing condition was maintained by adding nitric acid as needed. When digestion was complete, the cooled digest was treated with about 20 ml. of distilled water and evaporated to the fumes of SO_3. The resulting digest assumed a light yellow to colorless appearance.

The distillation was carried out as directed by Sultzaberger, i. e., by distilling the arsenic trichloride into 7.0 ml. of dilute nitric acid solution. Distillation was carried out in such a way that bubbles were formed at the rate of about one per second. Because the solution sometimes bubbled excessively, it was found advisable to insert the arm of the Fresenius flask into the mouth of the 50 ml. Erlenmeyer flask which was to be used for the color development. The distillate was then treated as directed, the heteropoly blue color was developed, and the per cent transmission read on a Cenco photelometer using the red filter.

This procedure was carried out for both the standardization curve and for the arsenic-containing biological material.

The standardization curve was prepared by adding known quantities of the standard arsenic solution to 0.2 - 0.5 g. samples of arsenic-free

laboratory chow. Blanks were determined for samples containing only acid as well as samples containing laboratory chow and acid. The results may be observed in Figure 2 and Table V.

In Figure 2, it will be noted that two curves are plotted. The lower curve was prepared by reading the arsenic samples with the photelometer set at 100 % transmission for distilled water. The red filter was used. If the photelometer with blank (organic matter + acid) is set at 100 % transmission, the correction factor for the arsenic in the chemicals is automatically made, as is indicated in the upper curve.

Recovery experiments were made on both biological materials and an organic arsenical compound. For the biological material a rat kidney homogenate was prepared and 5 ug. of As were added. In addition, a 20 % rat liver homogenate was prepared and 5 ug. of As were added. In the case of the organic compound, a neoarsphenamine (Merck, U. S. P. Lot No. 1236, containing 32.14 % As) solution (equivalent to 16 ug. of As/ml.) was prepared. The results are shown in Table VI.

TABLE V

STANDARDIZATION DATA FOR THE SULTZABERGER METHOD

Sample No.	Standard Sample	Std. Soln. 10 ug./ml.	% Trans. H_2O @ 100%	Org.+ Acid % Trans.
1	Blank (acid only)	None	91	-
2	Organic matter plus acid	None	93	100
3	Organic + 2 ug. As	0.2 ml.	88	95
4	Org. + 4 ug. As	0.4 ml.	86	93
5	Org. + 8 ug. As	0.8 ml.	80	87
6	Org. + 16 ug. As	1.6 ml.	68	75
7	Org. + 25 ug. As	2.5 ml.	54	61
8	Org. + 30 ug. As	3.0 ml.	50	57
9	Org. + 35 ug. As	3.5 ml.	44	51
10	Org. + 50 ug. As	5.0 ml.	31	38

TABLE VI

ARSENIC RECOVERY EXPERIMENTS BY THE SULTZABERGER METHOD

Sample Type	Sample Number	Per Cent Transmission	ug. As Recovered
0.5 ml. Neo-arsphenamine 16 ug./ml.	1	78	8.0
	2	78	8.0
	3	78	8.0
Kidney homogenate + 5 ug. As	1	82	5.5
	2	82	5.5
	3	83	5.2
20 % liver homogenate + 5 ug. As	3	80	7.0
	4	81	6.5

Results

Figure 2 shows a standardization graph in a quantitative range that should prove convenient for future research in this field. It is sensitive and reliable.

The data in Table VI show excellent recoveries from an organic material and good to fair recoveries from animal tissue homogenates. Further research is necessary to explain these variations in the tissue recoveries.

3. The Kingsley and Schaffert Method (12)

Reagents

The standard arsenic solution which was prepared according to Sultzaberger (11), contained 10 ug. of As per ml. Other reagents were made according to Kingsley and Schaffert. The stock iodine solution was standardized according to Kolthoff and Sandell (14).

Apparatus

The apparatus used was essentially the same as that described in the literature, except that rubber stoppered joints were used instead of ground glass joints.

Procedure

The standardization curve was prepared by adding known quantities of the standard arsenic solution to 0.2 - 0.5 g. of arsenic-free laboratory chow and submitting the samples to digestion with HCl. The arsenic was separated as arsine, which was trapped in 0.001 N I_2 solution. It was discovered that 2.5 ml. of the iodine solution did not provide sufficient volume for either the spectrophotometer or the photelometer cells; therefore, a volume of 5 ml. was used consistently for both the standardization curve and the samples.

The samples were digested with HCl for 15 minutes, cooled, treated with 10 ml. of HCl, and diluted to the original volume. They were then treated with 15 % KI and 40 % $SnCl_2$ and allowed to stand 15 minutes. In order to secure uniform generation of arsine, 3.0 g. of mossy zinc were added to each sample and generation was allowed to proceed for 1 hour.

The heteropoly blue colored complex was developed and read at 865 mu on the Beckman DU spectrophotometer. In addition, a calibration

curve was prepared for the Cenco photelometer using the red filter. The results are found in Figure 3 and Table VII. The readings were taken on both the Beckman and the Cenco with the instruments set at 100 % transmission for distilled water.

Recoveries were made on both the organic arsenical and biological materials. Triplicate samples were analyzed using 0.5 ml. of neoarsphenamine (8 ug. As) according to this procedure. The results are found in Table VIII.

Recovery experiments were also conducted on 5.0 ml. samples of a 10 % rat liver homogenate to which had been added 0.5 ml. of the standard arsenic solution containing 10 ug. of As per ml. The results are tabulated in Table IX. Duplicate blanks were determined on the homogenate according to the Sultzaberger method with a transmission of 92 % and 95 % on the Cenco.

TABLE VII

DATA FOR STANDARDIZATION CURVE

FOR THE KINGSLEY-SCHAFFERT METHOD

Sample Number	ug. As Added	% Trans. 865 mu	% Trans. Cenco
1	1	89.0	93
2	1	90.0	93
3	1	84.0	91
4	3	79.4	90
5	3	76.2	88
6	3	83.6	91
7	5	76.0	86
8	5	79.0	88
9	5	85.0	91
10	7	63.0	79
11	7	66.0	80
12	7	73.0	82
13	10	54.2	74
14	10	52.0	70
15	10	60.0	72

TABLE VIII

ARSENIC RECOVERY FROM NEOARSPHENAMINE

BY KINGSLEY-SCHAFFERT METHOD

Sample Number	ug. As in sample	Sample Volume	% Trans. 865 mu	% Trans. Cenco	ug. As Recovery
4	8	0.5 ml.	61.3	78	7.8
5	8	0.5 ml.	58.2	75	9.0
6	8	0.5 ml.	66.0	81	6.6

TABLE IX

ARSENIC RECOVERY FROM RAT LIVER HOMOGENATE

BY THE KINGSLEY-SCHAFFERT METHOD

Sample Number	ug. As in Sample	% Trans. 865 mu	% Trans. Cenco	ug. As Recovery
1-B*	None	-	92*	None
2-B*	None	-	95*	None
1	5	75.3	86	4.4
2	5	76.2	86	4.0

*The blank determined on this particular homogenate was analyzed only by the Sultzaberger method. It is valid, however, in indicating the lack of arsenic in the control rat.

Results

Figure 3 is the standardization curve for the Kingsley and Schaffert method, the data for which are found in Table VII. The curve obtained on the Beckman at 865 mu shows more scattering than that of the Cenco due to the increased sensitivity of the instrument.

Table VIII shows the recovery of arsenic from an organic arsenical. The average result is somewhat low. This may be due to a combination of technique and apparatus, since the arsenical easily undergoes oxidation.

Table IX likewise shows a low recovery which is accounted for under the discussion.

This procedure is apparently not the most satisfactory method for the micro determination of arsenic.

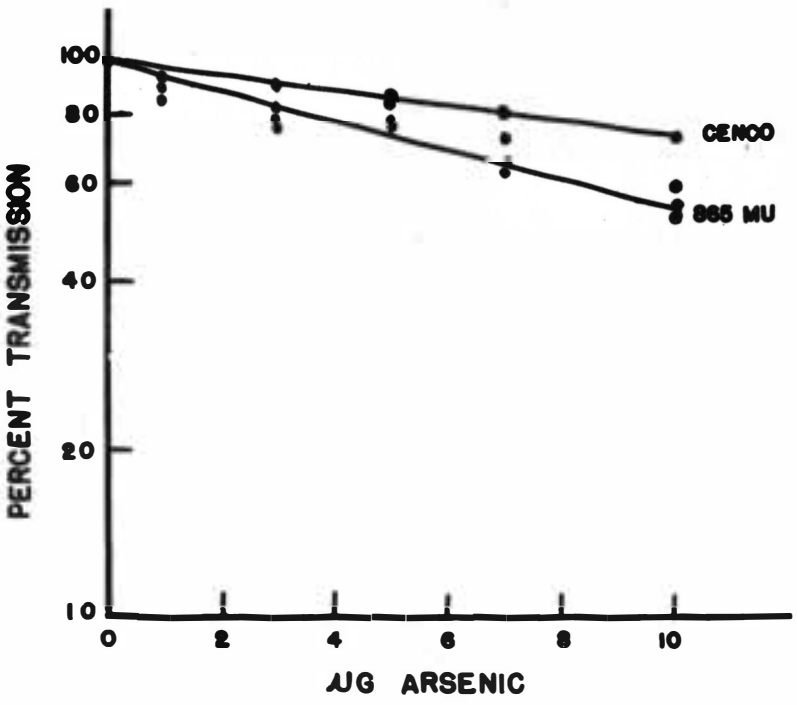

FIGURE 3

PLOT OF STANDARDIZATION CURVE FOR THE KINGSLEY AND SCHAFFERT METHOD

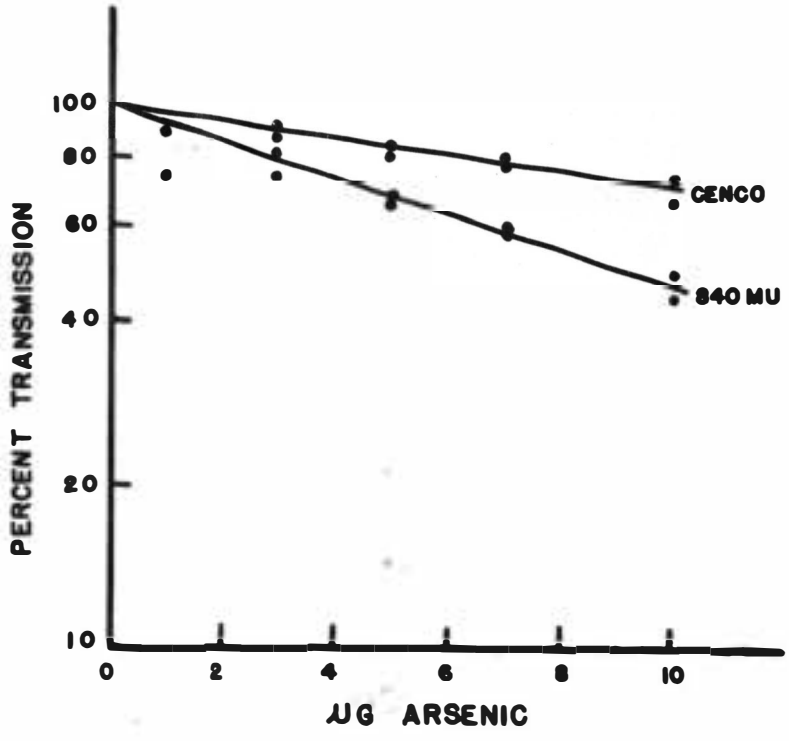

FIGURE 4

PLOT OF STANDARDIZATION CURVE FOR THE EVANS AND BANDEMER METHOD

4. The Evans and Bandemer Method (13)

Reagents

The reagents used conformed to those described in the literature (13) with the exception of the standard arsenic solution, which was prepared according to Sultzaberger (11).

Apparatus

Crucibles - A number 2 Coors crucible was used, since a number 4 was not available.

Apparatus for distillation - Rubber stoppered joints were used instead of the ground glass joints. All joints were water sealed.

Other apparatus conformed to that in the literature (13).

Procedure

A suitable quantity of the biological material was placed in a number 2 Coors crucible lined with ashless filter paper to which was added 6.0 ml. of saturated magnesium nitrate solution. After thorough mixing, the filter paper was folded over and the stirring rod wiped clean with a small piece of filter paper which was added to the contents of the crucible. The sample was then placed on a steam bath to evaporate any excess water. The contents of the crucible were then gently ashed using a small flame, after which the crucible was placed in a cold muffle furnace, the temperature raised to about 600°C. and allowed to remain over night.

Difficulty was encountered in the initial ashing, since the largest Coors crucible available was a number 2 instead of a number 4 as required. This limited the size of the sample to about 2.5 ml. of homogenate. Best results were obtained by gently heating the evaporated sample with a small Bunsen flame until all of the volatile material

was expelled; meanwhile, the expansion of the ash was carefully controlled, because biological material has a tendency to expand many times its original volume when subjected to heat under these conditions.

The cooled ash was then moistened with a few milliliters of distilled water, dissolved in 17.0 ml. of dilute HCl, and transferred to a 125 ml. Erlenmeyer flask. The crucibles were rinsed with 2 - 12 ml. portions of the HCl and the washings added to the flask.

The arsenic was separated as arsine using 3.0 g. of mossy zinc, and the arsine trapped in 5.0 ml. of 0.001 N I_2 solution. The heteropoly blue colored complex was developed and the per cent transmission read at 840 mu on the Beckman DU spectrophotometer.

The standardization curve was prepared by using arsenic-free laboratory chow for organic material, to which were added known quantities of the standard arsenic solution. Per cent transmission was determined on both the spectrophotometer and the photelometer. The results of the standard curve may be found in Figure 4 and Table X.

Recovery experiments were conducted using neoarsphenamine (16 ug. As/ml.) and rat liver homogenate. The samples were read against water. A 2.5 ml. sample of a 10 % rat liver homogenate was used to which was added 0.5 ml. of the standard arsenic solution (10 ug. As/ml.). The results are found in Table XI.

TABLE X

STANDARDIZATION CURVE DATA

BY THE EVANS AND BANDEMER METHOD

Sample Number	ug. As Added	% Trans. 840 mu	% Trans. Cenco
1	1	74.0	86
2	1	89.8	95
3	1	89.9	95
4	3	74.0	86
5	3	73.2	87
6	3	81.5	91
7	5	68.0	84
8	5	65.5	83
9	5	64.5	81
10	7	60.0	79
11	7	58.5	77
12	7	58.5	77
13	10	44.2	72
14	10	48.9	66
15	10	44.0	72

TABLE XI

ARSENIC RECOVERIES BY THE EVANS-BANDEMER METHOD

Sample Type	Sample Number	ug. As in Sample	% Trans. 840 mu	% Trans. Cenco	ug. As Recovered
Neoars-	8	8	53.0	76	8
phenamine	9	8	54.0	75	8
Liver	1-B*	None	-	92*	None
homogenate	2-B*	None	-	95*	None
	7	5	69.8	84	4.8
	8	5	64.5	83	5.3

*The blank on the 10 % rat liver homogenate was determined on the control rat by the Sultzaberger method.

Results

Figure 4 and Table X show the results of the calibration curve. The points are not too widely scattered providing a typical standardization curve. The variations that appear are due to technique as may be seen from Table XI.

Care must be exercised while handling the ash. The fritted gas dispersion cylinder is a source of error in arsine generation which error might be reduced to a minimum through constant application of the method.

5. The Evans-Bandemer Modification

Reagents

The reagents are the same as those used in the Evans-Bandemer (13) method with the following exceptions:

15 % KI solution was not required.

Dilute HCl solution was not required.

Approximately 12 N H_2SO_4 solution (prepared as required from 36 N arsenic-free H_2SO_4, reagent grade) was used instead of the HCl.

Apparatus

The apparatus used was essentially the same as that described for the Evans-Bandemer method with the following exceptions:

Rubber stoppered joints replaced the ground glass joints.

The coarse fritted gas dispersion cylinder was replaced by a glass delivery tube 3.5 mm. inside diameter with a capillary drawn out to about 0.5 mm. inside diameter at the end.

Crucible - A No. 2 Coors crucible was used for ashing, although a No. 4 crucible would greatly facilitate the procedure.

Procedure

The procedure followed for the modification was essentially the same as that followed for the Evans-Bandemer method up to the point of dissolving the ash.

The arsenic-containing biological material was placed in a No. 2 Coors crucible lined with an ashless filter paper. Six milliliters of a saturated magnesium nitrate solution were added and the mixture stirred. The filter paper was folded over with the aid of the stirring rod, and the rod was wiped clean with a small piece of filter paper which was added to the contents of the crucible.

The crucible and contents were placed on the steam bath for about 2 hours and the latter allowed to evaporate to dryness. The sample was gently heated to an ash with a Bunsen burner, using a small flame. Care must be exercised here, as the biological material will expand to many times its original volume. Also, the ash is light and a strong flame or draft will blow it out of the crucible. The sample was then placed in a cold muffle furnace, the temperature was raised to 600°C., and the sample was allowed to remain overnight.

The cooled ash was moistened with a few milliliters of distilled water, and 10 ml. of 12 N H_2SO_4 were added dropwise. The ash was dissolved by stirring and transferred to a 125 ml. Erlenmeyer flask. The crucible was washed with 2 - 10 ml. portions of 12 N H_2SO_4, and the washings were added to the contents of the Erlenmeyer flask.

The sample was then treated with 1.0 ml. of freshly prepared 40 % $SnCl_2$ solution and allowed to stand 15 minutes.

Meanwhile, the lead acetate filter was prepared as described in the literature (13), and 5.0 ml. of 0.001 N I_2 solution were placed in a 12 cm. test tube immersed in an ice bath.

Three grams of mossy zinc were added to the acid solution and the apparatus connected for distillation. Water seals were provided at all joints.

Generation was allowed to continue for one hour at the end of which time the iodine solution was removed.

The lead acetate filter was then removed, washed at once with about 10 ml. of concentrated nitric acid, and rinsed with about 150 ml. of distilled water.

The iodine solution containing the arsenic was treated first with

0.5 ml. of ammonium molybdate solution and then with 0.2 ml. of the hydrazine sulfate solution. The solution was thoroughly shaken after each addition. The heteropoly blue complex was developed by placing the test tube covered with a marble or glass bulb in a water bath at 80-90°C. for 10 minutes.

The standardization curve was prepared by adding known quantities of the standard arsenic solution to 0.2 - 0.5 g. of arsenic-free laboratory chow and following the procedure described.

The results for the standardization curve are found in Figure 5 and Table XII.

Recovery experiments were conducted using neoarsphenamine (16 ug./ml.), the results of which are shown in Table XIII.

A 2.5 ml. sample of a 10 % rat liver homogenate was used to which was added 0.5 ml. of the standard arsenic solution (10 ug. As/ml.). The blank on the liver was determined by the Sultzaberger method. Recoveries are shown in Table XIV.

TABLE XII

DATA FOR STANDARDIZATION CURVE

EVANS-BANDEMER MODIFICATION

Sample Number	ug. As Added	% Trans. 840 mu	% Trans. Cenco
1	0	93.8	96
2	0	92.5	97
3	3	78.0	90
4	3	78.0	90
5	5	66.0	83
6	5	70.8	86
7	7	59.8	80
8	7	60.8	80
9	10	47.0	72
10	10	--	--

TABLE XIII

ARSENIC RECOVERY FROM NEOARSPHENAMINE

BY THE EVANS-BANDEMER MODIFICATION

Sample Number	ug. As Added	ml. of Sample	% Trans. 840 mu	% Trans. Cenco	ug. As Recovered
1	3.2	0.2	78.5	89	3.0
2	3.2	0.2	81.0	90	2.6
3	4.8	0.3	70.5	85	4.4
4	4.8	0.3	68.0	83	5.2
5	9.6	0.6	56.0	77	8.0
6	9.6	0.6	55.8	77	8.0

TABLE XIV

ARSENIC RECOVERY FROM ANIMAL TISSUE

EVANS-BANDEMER MODIFICATION

Sample Number	ug. As Added	% Trans. 840 mu	% Trans. Cenco	ug. As Recovered
1-B*	None	-	92*	None
2-B*	None	-	95*	None
8	5.0	67.5	84	4.9
9	5.0	69.0	85	4.4

*The blank on the 10 % rat liver homogenate was determined by the Sultzaberger method.

Results

The Evans-Bandemer Modification provides an excellent calibration curve as is shown in Figure 5. Satisfactory results were also obtained on the Cenco photelometer. This instrument has the advantage of rapidity over the spectrophotometer and is still sufficiently accurate for this determination.

The sensitivity of the method requires more research. At present it is accurate to 1 ug. According to Tables XIII and XIV, a sensitivity of 0.1 ug. might be attained since the heteropoly blue color developed is quite intense. This has the disadvantage of limiting the maximum quantity of arsenic that might be analyzed.

The inclusion of the 40 % $SnCl_2$ in the procedure requires more research.

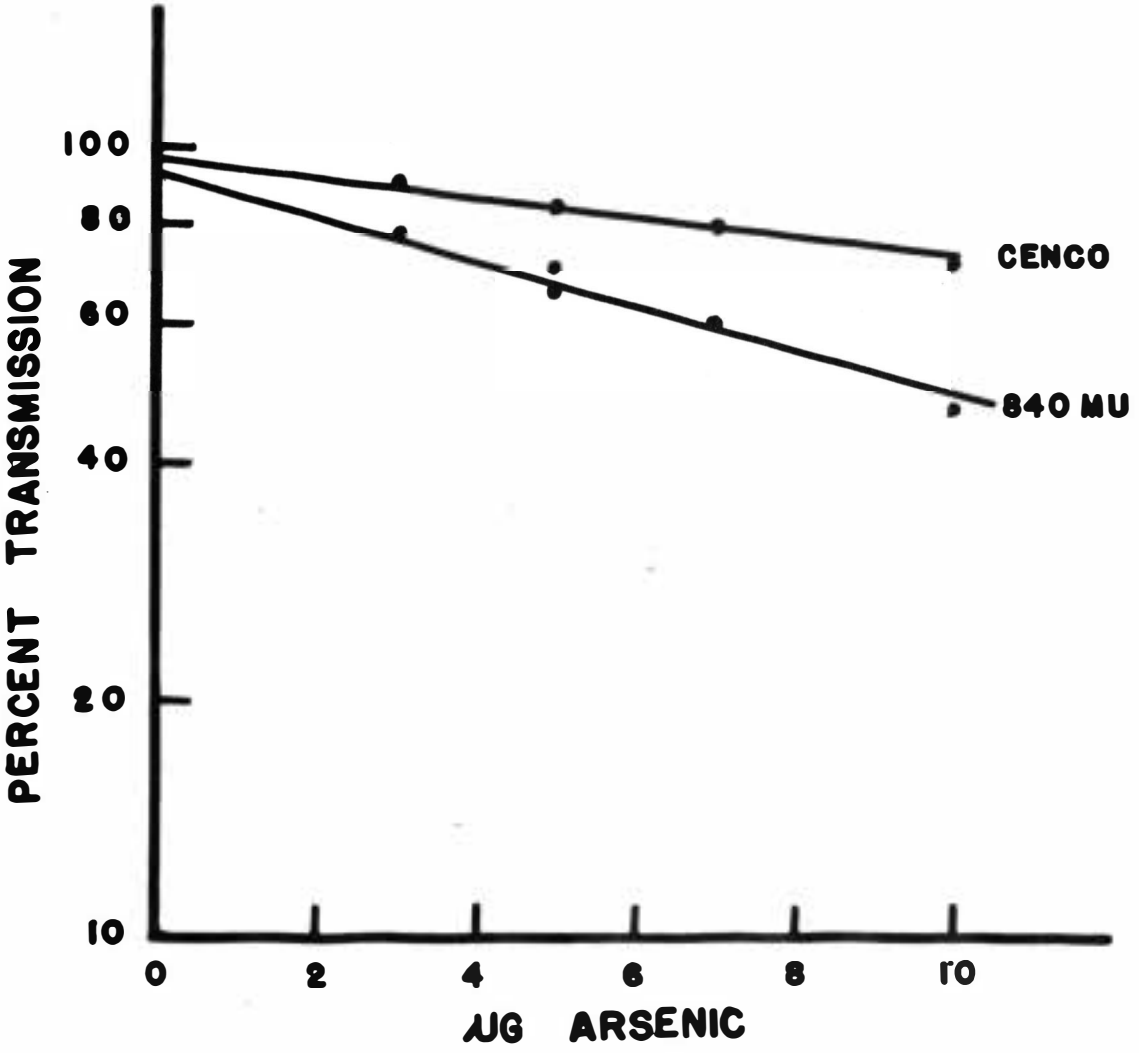

FIGURE 5

PLOT OF STANDARDIZATION CURVE FOR THE EVANS AND BANDEMER MODIFICATION

COMPARISON OF THE METHODS

1. Comparison of the Evans-Bandemer Modification with the Gutzeit and Kingsley methods.

A 592 g. male rat (albino), that had been drinking water containing 10 parts per million of arsenic as arsenic for six months, was sacrificed. The liver was removed and chilled to slow enzymatic action. A 20 % homogenate was prepared using a Waring blender. Analysis of the arsenic content of the liver homogenate was then made by the three methods. The results are shown in Table XV calculated as ug. As/g. of liver.

2. Comparison of the Gutzeit, Sultzaberger and Kingsley methods.

A 560 g. male rat that had been drinking arsenic water, 10 parts per million As, for a period of six months, was sacrificed and a 16.6 % liver homogenate prepared. Arsenic analyses were calculated as ug. of As/g. of liver. The results are shown in Table XVI.

3. Comparison of the Gutzeit, Kingsley, Evans-Bandemer methods, and the Evans-Bandemer modification.

Difficulty was encountered in obtaining sufficient biological material; therefore, it was decided to homogenize two arsenic-containing livers together.

Two young male rats weighing 370 g. and 380 g. respectively were sacrificed and a 20 % homogenate prepared by homogenizing the livers in a Waring blender. The results of the analyses are shown in Table XVII.

4. Comparison of the Sultzaberger and Evans-Bandemer Modification.

Due to erratic results obtained in Table XVII, it was decided to make a comparison analysis using the more dependable method (Sultza-

berger) as a control.

A young 370 g. male rat that had been on arsenic water (10 parts per million) for 3 months was sacrificed and a 20 % liver homogenate prepared.

Because undue enzymatic activity might alter results, the analytical equipment was set up ahead of time to prevent delay in analysis. Triplicate samples were analyzed according to both methods, the results of which are shown in Table XVIII.

For the Sultzaberger method, a 5 ml. sample of the homogenate was taken (1.0 g. liver); and for the Evans-Bandemer Modification, a 2.5 ml. sample of the homogenate (o.5 g. liver) was used .

TABLE XV

COMPARISON OF THE EVANS-BANDEMER, GUTZEIT, AND KINGSLEY METHODS

ug. arsenic per gram of liver

Sample Number	Gutzeit Method	Kingsley Method	Modification of E. & B.
1	2	6.4	14.0
2	2	10.0	12.8

TABLE XVI

COMPARISON OF THE GUTZEIT, SULTZABERGER, AND KINGSLEY METHODS

ug. arsenic per gram of liver

Sample Number	Gutzeit Method	Sultzaberger Method	Kingsley Method
1	4.2	3.5	4.2
2	4.2	4.2	2.2
3	-	-	2.2

TABLE XVII

COMPARISON OF THE GUTZEIT, KINGSLEY, EVANS-BANDEMER,

AND EVANS-BANDEMER MODIFICATION

ug. arsenic per gram of liver

Sample Number	Gutzeit* Method	Kingsley Method	Evans-Bandemer	E. and B. Modification
1	10	2.5	16.0	20.0
2	11	2.5	17.4	18.0
Ave. ug. As	10.5	2.5	16.7	19.0

*Length of stains in Gutzeit determination

Sample	Stain length	Ave. length
1	10/8 mm.	9 mm.
2	15/8 mm.	11 mm.

TABLE XVIII

COMPARISON OF THE SULTZABERGER AND EVANS-BANDEMER MODIFICATION

ug. arsenic per gram of liver

Sample Number	Method	% Trans. Cenco	Ug. As/1.0 g. Liver
1	Sultzaberger	72	12.0
2	"	71	12.5
3	"	71	12.5
4	E. and B.	62	2(6.2) - 12.4
5	Modification	61	2(6.4) - 12.8
6	"	63	2(6.0) - 12.0

Results

Tables XV, XVI, and XVII show a comparison of the various methods of arsenic analysis studied using biologically bound arsenic. The best results in recovery experiments together with those containing the bound arsenic appear to have been obtained by the Sultzaberger and Evans-Bandemer methods.

The choice of a control method with which to compare the modification was made on the basis that the Sultzaberger method provided digestion and separation of arsenic in an entirely different form from that of the modification, while the Evans-Bandemer did not. For example, in the Sultzaberger determination, the biological material is destroyed by wet combustion and the arsenic separated as $AsCl_3$. In the modification, the biological material is destroyed by ashing and the arsenic is separated as AsH_3.

DISCUSSION

1. The Gutzeit Method

There are many points of criticism involving the Gutzeit method of arsenic analysis, perhaps the most important being that it is time-consuming in each step of the operation. Large samples, which lengthen the digestion time, are required for micro quantities of arsenic. Digestion requires on the average from four to eight hours, depending upon the size of the sample and the type of material. In turn, this means large quantities of nitric acid must be consumed.

The intermediate steps are time-consuming, the generation of arsine being one of the more tedious steps of the procedure. The zinc must be arsenic-free and of uniform quality to insure even generation and absorption of the arsine on the indicator paper. It may be mentioned that the used spongy zinc will trap arsenic on its surface, as was determined later by adding more HCl and zinc shot. The results were not included in this paper.

Arsine evolution was found to be uneven, as is shown in Table XVII and by Saterlee et al., (15). The stains were measured on one side of the mercuric bromide paper and found to be 15 mm. in length, while on the reverse side they were 8 mm. in length. In addition, the extent of the reaction between the arsine and the mercuric bromide varied, as is indicated by the shade of the stain. This may be observed in a typical plate by Scott (16).

The Gutzeit method is limited to semi-micro quantities, the optimum being between 0.02 and 0.03 mg. of arsenic (9).

The technique employed by the analyst must be consistent without fail and can be attained only after much experience which the average

investigator will not develop unless he is making Gutzeit determinations daily. Clark (17) states that even then one cannot be sure of the results.

For these reasons the author has deemed it inadvisable to consider the Gutzeit method as being reliable for the determination of micro quantities of arsenic.

2. The Sultzaberger Method

This method was developed by incorporating the ideas of several investigators and coming up with a simple, rapid, accurate micro method of arsenic analysis. It is based on the separation of arsenic as the trichloride and the subsequent development of the molybdenum-arsenic complex blue color. The method has several advantages, even though there are some disadvantages.

The sample is small and may be rapidly digested in comparison with the Gutzeit method. The separation of the arsenic is very rapid and easy to carry out. The apparatus is simple and cheap, with perhaps the exception of the Fresenius flask, and may be found in any high school chemistry laboratory.

Technique is of importance. Charring of the digest should be avoided since loss of arsenic may occur (18). Distillation must be observed closely as is indicated by Sultzaberger. However, this is not too difficult, as the absorbing solution ceases to bubble when all of the $AsCl_3$ has distilled.

The most difficult part of the operation occurs in the evaporation of the absorbing solution. Here the temperature must be rigidly controlled, or arsenic loss will occur. It was found that it was

better not to evaporate to dryness on the hot plate, but to evaporate the last 3 - 5 ml. of liquid in an oven set at 120-125°C. This step requires strict attention and is the chief source of error in the method.

In development of the color it is necessary to cover the containers to prevent evaporation of the liquid, which, of course, would alter the intensity of the color.

Consistent results, as indicated in Table VI, were obtained by the author after learning the technique. The method is satisfactory for micro quantities of arsenic.

It was noted that in digestion the perchloric acid is not necessary as is used by Morris and Calvery (19). This was eliminated by the author because of the danger involved.

3. The Kingsley and Schaffert Method

This method is notable chiefly for its rapidity of digestion. The Sultzaberger method usually requires two to four hours for digestion of the biological material, while the method in question requires only fifteen minutes.

Recovery experiments, and arsenicals easily undergoing oxidations, will respond to this type of digestion, as may be observed in Tables VII and VIII. Some recoveries have been low, a typical example of which is found in Table IX. This may have been due to faulty technique rather than to the digestion procedure. Biologically bound arsenic, however, produced erratic results, as may be seen in Tables XV, XVI, and XVII. The author feels that low results were obtained due to incomplete breakdown of the biological material. This is substantiated

by the work of Evans and Bandemer (13).

In addition, the author does not agree with Kingsley in the preparation of the calibration curve. It is the author's opinion, formed through experience, that the standardization curve should be determined by subjecting the sample to the same treatment as that to which biologically-bound arsenic would be subjected in order to give a true picture of the method.

Another possible source of error is the fritted gas dispersion cylinder. It was found later that a capillary would suffice, but in order to reproduce the original work, the cylinder was used for all experiments indicated. The gas dispersion cylinder will retain some of the arsenic-containing solution which cannot be removed by air pressure. Washing would be out of the question, since the added liquid (either water or iodine) would alter the volume and, in turn, the color intensity developed.

Because of these objections, the Kingsley-Schaffert method, in the opinion of the author, is not a satisfactory method of analysis of arsenic in biological material.

4. The Evans and Bandemer Method and the Modification.

The essential difference between the Evans-Bandemer and Kingsley-Schaffert methods is in the liberation of the bound arsenic from the biological material. The Evans-Bandemer method depends upon the destruction of the biological material by ashing and retaining the arsenic, probably as the magnesium pyoarsenate ($Mg_2As_2O_7$).

There are some criticisms of the method which were worth investigation. For example, ashing required a little longer period than does

the digestion by the Sultzaberger method, but it does not require the attention of the analyst as does the Sultzaberger method.

A possible source of arsenic loss is again the fritted gas dispersion cylinder, the same as is found in the Kingsley method. The author therefore attempted a modification of the Evans-Bandemer method by dissolving the ash in 12 N H_2SO_4 and treating the solution with 1 ml. of 40 % $SnCl_2$. The fritted gas dispersion cylinder was replaced with a capillary tube.

The color developed seemed to be a little more intense than that of the Evans-Bandemer method. Colorimetric readings were made on both the Beckman DU spectrophotometer and the Cenco photelometer. The spectrophotometer is slower in operation but gives a more sensitive reading. However, this disadvantage may be offset by using a later model colorimeter and neither speed nor accuracy will be sacrificed.

The author feels that it was not necessary to include the 1.0 ml. of 40 % $SnCl_2$, since it is not essential to reduce the arsenic to the trivalent state prior to distillation, as is pointed out by Magnuson and Watson (20). They showed that in order for the color to be developed the arsenic must be in the pentavalent form. It therefore seemed logical that the arsenic could be distilled without reduction and the color developed directly. This was done in a few instances; however, not enough trials were conduoted to substantiate the idea.

SUMMARY

Five selected methods for the quantitative micro analysis of arsenic in biological materials have been compared experimentally and evaluated on the criteria of accuracy, precision, speed and simplicity of equipment and laboratory techniques. None of the methods has proven to be consistently reliable in the preparation of standardization data, in the analysis of an organic arsenical, or in the analysis of biologically-bound arsenic.

A modification of the Evans-Bandemer method of arsenic determination in biological materials, based on the formation of the heteropoly blue colored complex is presented. The procedure is accurate to one microgram with an optimum range of one to twenty micrograms. This modification and the Sultzaberger method were found to be the two most reliable procedures tested in this laboratory.

The technique and results of each method have been critically reviewed in the discussion.

THE COURSE OF FUTURE RESEARCH

The problem of arsenic analysis in biological material is still open to research. The chief source of error appears to be in digestion of the sample.

Digestion of the sample has created much dissention among investigators. Questions have arisen concerning the loss of arsenic by charring the digest, and the necessity of strong oxidizing agents and their effects. Consequently extraction procedures have been attempted but with only limited success.

The majority of micro methods of arsenic separation have been based on the volatility of the arsenic in one form or another. Beside those errors that may be contributed by the analyst, others may arise as a result of incomplete separation of arsenic. The ideal method, of course, would involve a direct determination without separation. This cannot be realized by conventional methods because of interfering ions such as PO_4^{-3}, VO_3^{-}, Fe^{+3}, Al^{+3}, and Ce^{+4}.

Polarography could be the answer. This would be disregarding the criteria, however, of simple laboratory techniques and equipment. The polarograph is not available to all workers and many technicians are not trained in the use of the instrument.

More research is required concerning the chemical composition of the heteropoly blue complex, not to mention the effect of enzyme activity on the loss of arsenic.

Development of better and more sensitive methods of arsenic analysis would permit exploration of many pathways, especially in the fields of pharmacology and intermediary metabolism. However, before these pathways may be defined with confidence, the arsenic must be followed quantitatively.

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